Mechanisms of 2-Butoxyethanol Carcinogenicity: Studies on Syrian Hamster Embryo (SHE) Cell Transformation

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Abstract

Previous studies showed that 2-butoxyethanol increased liver tumors in B6C3F1 mice following chronic exposure. While the mechanism of 2-butoxyethanol-induced liver carcinogenicity has not been defined, 2-butoxyethanol has been shown to induce hemolysis in rodents via 2-butoxyacetic acid, the major metabolite of 2-butoxyethanol. This toxic effect, coupled with the observation that continued treatment with 2-butoxyethanol results in hemosiderin deposition in the liver, has led to our hypothesis that liver carcinogenicity by 2-butoxyethnaol is mediated via oxidative stress (iron catalyzed) and Kupffer cell activation. The present study used Syrian Hamster Embryo (SHE) cell transformation, a surrogate in vitro model for carcinogenesis in vivo , to examine whether 2-butoxyethanol, 2-butoxyacetic acid, or iron (ferrous sulfate) produced cell transformation. SHE cells were treated with either 2-butoxyethanol (0.5–20 mM), 2-butoxyacetic acid (0.5–20 mM), or ferrous sulfate (0.5–75 µg/ml) for 7 days. 2-Butoxyethanol and 2-butoxyacetic acid did not induce cellular transformation. In contrast, treatment with ferrous sulfate (2.5 and 5.0 µg/ml) increased morphological transformation. Cotreatment of ferrous sulfate with the antioxidants α-tocopherol (vitamin E) or (-)-epigallocatechin-3-gallate (EGCG) prevented ferrous sulfate-induced transformation, suggesting the involvement of oxidative stress in SHE cell transformation. The level of oxidative DNA damage (OH8dG) increased following ferrous sulfate treatment in SHE cells; additionally, using single cell gel electrophoresis (comet assay), ferrous sulfate treatment produced an increase in DNA damage. Both

DNA lesions were decreased by cotreatment of ferrous sulfate with antioxidants. These data support our proposal that iron, produced indirectly through hemolysis, and not 2-butoxyethanol or its metabolite 2-butoxyacetic acid, is responsible for the observed carcinogenicity of 2-butoxyethanol.

Key words

2-Butoxyethanol (ethylene glycol monoalkyl ether) is a solvent with varied industrial and consumer uses. 2-Butoxyethanol induced an increase in both liver hemangiosarcomas and hepatocellular carcinomas in male B6C3F1 mice following chronic exposure (NTP, 2000), and it appears to be nonmutagenic based on short-term mutagenesis studies (Elliott and Ashby, 1997; Gollapudi et al., 1996; Hoflack et al., 1995). However, Hoflack et al. (1997) reported that 2-butoxyethanol inhibited methyl-methanesulfonate-induced poly(ADP-ribosyl) synthesis, the substrate of poly(ADP-ribosyl) polymerase, and suggested alteration of DNA repair in the cells treated with genotoxic substances, but 2-butoxyethanol was without a genotoxic effect.

2-Butoxyethanol exposure results in hemolysis (Ghanayem and Sullivan, 1993; Ghanayem et al., 1992), attributed to 2-butoxyacetic acid, a major metabolite of 2-butoxyethanol (Ghanayem et al., 1987b,c). An additional finding following chronic treatment of male rats and mice was the accumulation of hemosiderin, indicative of iron accumulation, in the liver (Ghanayem et al., 1987a; Krasavage, 1986; NTP, 2000). Hepatic accumulation of iron has been associated with the induction of liver cancer (Niederau et al., 1985). These findings have led to the proposal that the increase in iron deposition via hemolysis by 2-butoxyacetic acid results in an increase in liver oxidative stress (via the generation of hydroxyl radicals by Fenton reactions), and ultimately leads to the observed neoplastic response in mouse liver. Oxidative damage arising from reactive oxygen species and other free radicals includes lipid peroxidation, oxidation of proteins, and oxidative damage to DNA. Oxidative damage has been linked to a number of human diseases including carcinogenesis (Perera et al., 1987; Toyokuni, 1996). The hydroxyl radical can mediate the formation of 8-hydroxydeoxyguanosine (OH8dG) from deoxyguanosine (Kasai and Nishimura, 1984). An association between OH8dG and carcinogenesis (Kasai, 1990) and cell transformation (Zhang et al., 2000) has been established. Furthermore, the production of oxidative DNA damage from iron overload in the liver has been suggested as a mechanism in hemochromatosis, a genetic disease associated with iron overload (Kang et al., 1998).

Syrian Hamster Embryo (SHE) cellular transformation shares similarities with multistage carcinogenesis in vivo (Barrett et al., 1984). The formation of morphological transformation is an early event in the multistep process of SHE cell transformation and correlates with carcinogenesis in vivo. Thus, the appearance of morphologically transformed SHE cells has been used as a surrogate for carcinogenesis in vivo. SHE cell transformation has been used to screen the carcinogenic potential of many chemical agents and has a high concordance to the carcinogencity of these chemicals (Isfort et al., 1996). SHE cell morphological transformation has also been utilized to study underlying mechanisms of genotoxic and nongenotoxic chemical carcinogenesis (Barrett et al., 1984; Isfort et al., 1994). In the present study, the SHE cell transformation model was used to examine the possible mode of action of 2-butoxyethanol–induced carcinogenesis. To examine the proposal that the liver neoplasia induced by 2-butoxyethanol in the mouse is the result of oxidative stress, produced indirectly from the production of hemolysis and resulting iron overload in the liver, we examined if 2-butoxyethanol, its metabolite 2-butoxyacetic acid, or ferrous sulfate (to mimic iron deposition) induced SHE cell transformation. We also examined the potential contribution of oxidative stress in the induction of cellular transformation by using the antioxidants α-tocopherol (vitamin E) and EGCG and by examining OH8dG levels as well as DNA strand breaks in treated SHE cells.

MATERIALS AND METHODS

Materials.

2-Butoxyethanol and ferrous sulfate ($FeSO_4$) were purchased from Sigma Chemical Co. (St. Louis, MO); 2-butoxyacetic acid was purchased from Spectrum Chemicals (Gardena, CA). Certificates of analysis indicated >99% purity for 2-butoxyethanol, 2-butoxyacetic acid, and $FeSO_4$. Le Boeuf's Dulbecco's Modified Eagles Medium (DMEM) was purchased from Quality Biological Inc. (Gaithersburg, MD)., fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT), and L-Glutamate was purchased from Gibco (Grand Island, NY). Vitamin E (d-α-tocopherol acetate) and all other chemicals used were from Sigma Chemical Co. (St. Louis, MO).

SHE cell isolation and culture.

SHE cells were isolated and cultured as described (Kerckaert et al., 1996). Briefly,

primary embryo cells were isolated from 13-day gestation Syrian golden hamsters (Charles River, Wilmington, MA), cryopreserved, and cultured in DMEM supplemented with 20% FBS and 4 mM L-glutamate at 37°C in 10% CO_2 and 90% relative humidity. A feeder layer was prepared by plating 2×10^6 SHE cells into 30 ml complete media in a T-150 tissue culture flask. After 48 h, the cells were detached with 0.05% trypsin, 0.02% Na_2EDTA in Ca^{+2}, Mg^{+2}-free Hank's Balanced Salt Solution. The cells were irradiated (5,000 rads), on ice (40 min), and plated (4×10^4/60 mm culture dish in 2 ml of medium). The target cells were prepared by plating 1×10^6 thawed cells per T-25 tissue culture flask containing 5 ml of complete media. After 24 h, the cells were detached and plated onto the feeder layer at a density of 80 cells/60 mm culture dish with 2 ml of medium. After 24 h, cell cultures were exposed to test compounds for 7 days. For 2-butoxyacetic acid treatment, the pH was adjusted to 6.7 with 1N NaOH. Following 7 days of treatment, the cultures were rinsed, methanol fixed, and stained with Giemsa (Sigma, St. Louis, MO). Transformation was scored by two individuals, independently (without knowledge of treatment groups), using a Nikon stereoscopic zoom microscope. A morphologically transformed colony was defined as a colony exhibiting a crisscrossed, multilayered, irregular pattern of growth (Kerckaert et al., 1996). For each group, total colony number, morphological transformation frequency ([the number of transformed colonies/total number of colonies scored] × 100), and relative plating efficiency [RPE, (test group plating efficiency/solvent control plating efficiency) × 100] were determined.

Determination of OH8dG.

OH8dG was measured using total DNA isolated from treated and control SHE cells. DNA was isolated by sodium iodide (NaI) chaotropic extraction (Wang et al., 1994). 72 h prior to treatment, cells were seeded (2×10^5/100 mm culture plates). Cells were then exposed to 2-butoxyethanol, 2-butoxyacetic acid, or $FeSO_4$ (3 dishes/each treatment) for 24 h. After treatment, the media was removed, and the cells were washed with PBS containing 0.1 mM desferol, detached, lysed with Triton x-100 buffer, and centrifuged

(10,000 × g, 20 s). The homogenates were digested sequentially with proteinase K (10 U, 30 min, 37°C) and RNase A (5 U, 10 min, 37°C). Following digestion, DNA was precipitated with NaI (0.2M) and ice-cold isopropyl alcohol, and centrifuged (10,000 × g, 10 min). DNA was dissolved in Tris-HCl (10 mM; 200 µl) and digested with nuclease P1 (10 U, 30 min, 37°C) and alkaline phosphatase (14 U, 60 min, 37°C). After centrifugation (10,000 × g, 10 min), 200 µl of supernatant was used for analysis of OH8dG using HPLC with electrochemical detection. Elution was with a mobile phase consisting of 100 mM of sodium citrate (pH 5.2; 1.0 ml/min) on a Waters Nova-Pak C_{18} reversed phase analytical column on a Waters 2690 Alliance HPLC System (Waters, Milford, MA). OH8dG was detected electrochemically (ESA Coularray, 12 channel; ESA, Chelmsford, MA) and 2`-deoxyguanosine (dG) detected at 250 nm (Waters™ 996 system; Waters, Milford, MA). OH8dG and dG were quantitated from standards prepared immediately prior to sample analysis.

Detection of DNA strand breaks by single cell gel electrophoresis (comet) analysis.

To detect DNA strand breaks, alkaline lysis was used, followed by alkaline single cell gel electrophoresis (comet assay). Forty-eight h prior to treatment, SHE cells were seeded (2×10^5 in 60 mm culture plates) in 5 ml Le Boeuf's DMEM with 20% FBS and 4mM L-glutamate, and incubated (37°C; 10% CO_2; 90% humidity). Cells were exposed to 2-butoxyethanol, 2-butoxyacetic acid, or $FeSO_4$ for 24 h. After treatment, cells were washed (2 ml of HBSS; 2×), detached (0.002% trypsin, 0.0004 % EDTA.4Na, 1 ml, 2 min, 37°C). Following detachment, the comet assay was performed as described (Rojas et al., 1999; Tice, 1995). Briefly, cells were suspended (1×10^4) in 1% low melting agarose (LMA) and spread on frosted slides. After solidification, a 80 µl layer of LMA was spread over the cells. The slides were then immersed in lysis solution (1% Triton X-100, 10 % DMSO, 2.5M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0, 1 h, 4°C) and electrophoresed in buffer (1 mM Na_2EDTA and 0.3M NaOH, pH > 13.5) at 25 V, 300 mA

for 30 min at 4°C. After electrophoresis, the slides were rinsed (3×, 0.4M Tris, pH 7.5), stained with ethidium bromide (60 µl; 20 µg/ml), and covered with cover slips. A total 100 nuclei/data point (50 randomly chosen cells per slide) were measured with a Nikon fluorescence microscope. Images were digitized (NIH image system) and DNA damage was expressed as tail moment (fraction of DNA density of tail multiplied by tail distance; Rojas et al., 1999; Tice, 1995). Two slides were prepared from each group.

Statistics.

Transformation was considered significant at $p < 0.05$, by a one-tailed Fisher's exact test conducted on pooled data (n = 40 dishes) from two or more trials (Armitage, 1971). All other data were analyzed by one-way ANOVA followed by Duncan's test. Treatment groups were considered statistically different at $p < 0.05$.

RESULTS

Effect of 2-Butoxyethanol, 2-Butoxyacetic Acid, and FeSO$_4$ on SHE Cell Morphological Transformation

The effect of 2-butoxyethanol on morphological transformation in SHE cells is shown in Table 1. 2-Butoxyethanol was tested at concentrations ranging from 0.5 to 20 mM with 7-day exposure. No significant increase in morphological transformation frequency relative to control (media) was observed with 2-butoxyethanol treatment in SHE cells. The positive control (benzo(a)pyrene) produced an increase in transformation frequency in a range consistent with historical results. A dose-related increase in toxicity, as evidenced by a reduction in relative plating efficiency (RPE, lower than 50%; Kerckaert et al., 1996) was seen with concentrations above 10 mM 2-butoxyethanol.

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TABLE 1
Effect of 2-Butoxyethnol on SHE Cell Transformation (7 Days)

The major metabolite of 2-butoxyethanol, 2-butoxyacetic acid, which is responsible for

the observed hemolysis seen in rats and mice, was also examined for SHE cell morphological transformation at concentrations ranging from 0.5 to 20 mM for 7 days (Table 2). Similar to 2-butoxyethanol, 2-butoxyacetic acid failed to induce a significant increase in morphological transformation over control values at the concentrations tested. Benzo(a)pyrene was positive for transformation as expected.

TABLE 2
Effect of 2-Butoxyacetic Acid on SHE Cell Transformation (7 Days)

The effect of ferrous sulfate (to mimic the iron deposition in the liver resulting from hemolysis induced by 2-butoxyacetic acid) on morphological transformation was also examined (Table 3). A significant increase in morphological transformation was seen following treatment with at 2.5 and 5.0 µg/ml ferrous sulfate for 7 days. Cellular transformation observed at 2.5 µg/ml $FeSO_4$ was the greatest (0.71%) of all concentrations tested. However, at concentrations above 10 µg/ml, $FeSO_4$ was cytolethal, as evidenced by a reduction in relative plating efficiency (Table 3).

TABLE 3
Effect of Ferrous Sulfate on SHE Cell Transformation (7 Days)

Effect of Cotreatment of Ferrous Sulfate with Antioxidants (EGCG or Vitamin E) on SHE Cell Morphological Transformation

Ferrous sulfate was coincubated with either the water-soluble green tea antioxidant EGCG (Fig. 1) or vitamin E (Fig. 2) to examine whether coincubation with antioxidants was protective against iron-induced SHE cell transformation. EGCG was used at concentrations ranging from 5 to 50 µM. EGCG itself had no effect on morphological transformation (Fig. 1). As shown previously, 2.5 µg/ml $FeSO_4$ produced a significant increase in morphological transformation. A significant reduction in ferrous sulfate-induced morphological transformation was observed following coincubation of 2.5 µg/ml $FeSO_4$ with either 25 or 50 µM EGCG (Fig. 1).

FIG. 1.

Effect of cotreatment of ferrous sulfate and EGCG on SHE cell transformation (7 days). SHE cells were cotreated with ferrous sulfate and EGCG for 7 days and incubated at 37°C in 10% CO_2 and 90% relative humidity. The cultures were then rinsed, fixed with methanol, stained with Giemsa, and evaluated for morphological transformation. *Statistically different from control, $p < 0.05$. **Statistically different from ferrous sulfate treatment, $p < 0.05$, Fisher's Exact Test.

FIG. 2.

Effect of cotreatment of ferrous sulfate and vitamin E on SHE cell transformation (7 days). SHE cells were cotreated with ferrous sulfate and vitamin E for 7 days and incubated at 37°C in 10% CO_2 and 90% relative humidity. The cultures were then rinsed, fixed with methanol, stained with Giemsa, and evaluated for morphological transformation. *Statistically different from control, $p < 0.05$. **Statistically different from ferrous sulfate treatment, $p < 0.05$.

Similarly, the effect of vitamin E on ferrous sulfate-induced morphological transformation was examined (Fig. 2). Vitamin E itself did not alter morphological transformation; however, a significant reduction in ferrous sulfate-induced morphological transformation was seen at concentrations of 150 µM vitamin E and higher (Fig. 2).

Effect of Ferrous Sulfate on DNA Damage Using the Alkaline Comet Assay

To examine whether DNA strand breaks were induced by ferrous sulfate, single cell gel electrophoresis (the comet assay) was performed (Fig. 3). SHE cells treated with ferrous sulfate (0.5, 1.0, and 2.5 µg/ml) for 24 h showed a concentration-related increase in DNA damage (comet tail moment). A significant increase in comet tail moment was evident following treatment with both 1.0 and 2.5 µg/ml ferrous sulfate. Coincubation of 2.5 µg/ml ferrous sulfate with the antioxidants vitamin E (200 µM) or EGCG (25 µM) prevented the DNA strand breaks induced by ferrous sulfate (Fig. 3).

FIG. 3.

Effect of cotreatment of ferrous sulfate and antioxidants on DNA strand breaks. SHE

cells were cotreated with ferrous sulfate and antioxidants for 24 h and incubated at 37°C in 10 % CO_2 and 90% relative humidity. The cultures were then rinsed, detached, and applied to single cell gel electrophoresis. One hundred comets/treatment were analyzed. The result was expressed as a Tail moment. *Statistically different from control, $p < 0.05$. **Statistically different from ferrous sulfate treatment, $p < 0.05$.

Effect of Ferrous Sulfate on OH8dG Formation

The formation of OH8dG by ferrous sulfate treatment in SHE cells was examined (Fig.

4). SHE cells treated with 0–5.0 µg/ml ferrous sulfate for 24 h resulted in a

concentration-related increase in OH8dG. A significant increase in oxidative DNA

damage was seen following treatment with 2.5 µg/ml ferrous sulfate and higher.

Coincubation of 2.5 µg/ml ferrous sulfate with the antioxidants vitamin E (200 µM) and

EGCG (25 µM) decreased OH8dG levels to control levels (Fig. 4).

FIG. 4.
Effect of cotreatment of ferrous sulfate and antioxidants on oxidative DNA damage. SHE cells were cotreated with ferrous sulfate and antioxidants for 24 h and incubated at 37°C in 10% CO_2 and 90% relative humidity. The cultures were then rinsed and detached. DNA was isolated, hydrolyzed, and applied to HPLC with EC detection for OH8dG analysis, n = 6. *Statistically different from control, $p < 0.05$. **Statistically different from ferrous sulfate treatment, $p < 0.05$.

DISCUSSION

SHE cell morphological transformation was used to examine the potential mechanism(s) involved in the carcinogenicity of 2-butoxyethanol. Based on previous studies that showed that 2-butoxyethanol and its major metabolite, 2-butoxyacetic acid, are not mutagenic, and that 2-butoxyacetic acid induced red blood cell hemolysis, we hypothesized that the carcinogenicity of 2-butoxyethanol was indirectly due to iron overload resulting from hemolysis and subsequent production of reactive oxygen intermediates. Treatment of SHE cells with 2-butoxyethanol or 2-butoxyacetic acid did not induce morphological transformation in SHE cells, confirming that the induction of cell transformation/carcinogenicity by 2-butoxyethanol was not a direct result of the parent chemical or the major metabolite. These negative results on cell transformation are in agreement with those previously reported in another system for 2-butoxyethanol and 2-butoxyacetic acid (Elliott and Ashby, 1997).

Iron, through the Fenton reaction, can generate hydroxyl radicals (Henle et al., 1996; Imlay and Linn, 1988; Imlay et al., 1988). Reactive oxygen species and other free radicals are known to participate in the carcinogenesis process (Perera et al., 1987; Toyokuni, 1996). Iron overload in humans, whether from genetic disease or from dietary intake, has been associated with the induction of hepatic cancer (Mandishona et al., 1998; Niederau et al., 1985; Stevens et al., 1994). 2-Butoxyethanol, through metabolism by alcohol and acetaldehyde dehydrogenases, results in the formation of 2-butoxyacetic acid, which subsequently induces red blood cell hemolysis. 2-Butoxyethanol-induced hemolysis occurs both in vivo and in vitro and is associated with a deposition of hemosiderin (iron deposition) and free hemoglobin in Kupffer cells and hepatocytes (Ghanayem and Sullivan, 1993; Ghanayem et al., 1987b,c; Krasavage, 1986). In the present study, treatment with ferrous sulfate (to mimic the iron deposition that would result from 2-butoxyethanol administration in vivo) resulted in a dose-related increase in morphological transformation frequency SHE cells. 2-Butoxyethanol exposure in vivo results in hemolysis up to approximately 20%. Based on the amount of iron in blood, 20% hemolysis is predicted to result in the liberation of 0.1 mg iron/ml of blood. Thus, the concentrations of iron used in the present study appear to be well within the predicted range expected following 2-butoxyethanol–induced hemolysis. However, the actual amount of free iron produced following hemolysis is not known. Further studies are necessary to determine free iron concentrations following hemolysis.

Cellular transformation has also been shown to occur in SHE cells exposed to divalent iron-containing minerals (Elias et al., 1995). The morphological transformation seen with these minerals was prevented upon coincubation with an iron chelator, further substantiating that the iron component of the minerals was mediating the transformation process. In addition, enhancement of viral transformation of SHE cells was seen following treatment with $FeCl_2$ and $FeSO_4$ but not by a ferric compound (Fe_2O_3; Castro et al., 1979; DiPaolo and Castro, 1979). Our results, showing the induction of morphological transformation in SHE cells with ferrous sulfate ($FeSO_4$) treatment, are in agreement with these studies.

Oxidative stress has been shown to participate in the carcinogenesis process (Kensler and Thrush, 1986, Kensler et al., 1989). The effect of 2-butoxyethanol and 2-butoxyacetic acid on oxidative stress and damage was previously examined in cultured mouse and rat hepatocytes (Park et al., 2002). These studies demonstrated that neither 2-butoxyethanol nor 2-butoxyacetic acid induced oxidative DNA or lipid damage in hepatocytes. Iron, however, significantly increased oxidative stress in both rat and mouse hepatocytes while only producing oxidative DNA damage in mouse hepatocytes. These results were consistent with the current negative results obtained on SHE cell transformation by 2-butoxyethanol and 2-butoxyacetic acid and further

suggest a role for iron in 2-butoxyethanol–induced liver cancer.

Several reports have documented the promotion or enhancement of carcinogenicity by iron in vivo. Iron was shown to enhance estrogen-induced renal tumors, diethylnitrosamine-initiated liver tumors, dimethylhydrazine-induced colon tumors, hexachlorobenzene-caused hepatic neoplasia, and benzoyl peroxide-mediated skin tumors (Carthew et al., 1997; Rezazadeh and Athar, 1998; Siegers et al., 1988; Smith et al., 1993; Wyllie and Liehr, 1998). Thus, the proposal that iron is involved in the mechanisms of 2-butoxyethanol–induced cell transformation/carcinogenicity, either as a direct mediator or indirectly through tumor promotion, is feasible. Oxidative stress and enhanced cell proliferation have also been implicated in the underlying mechanisms of iron promotion of carcinogenesis. That antioxidant (EGCG or vitamin E) coexposure prevented the morphological transformation observed in the present study further suggests an involvement of oxidative stress in ferrous sulfate-induced cellular transformation. The water-soluble antioxidant EGCG, a component in green tea, was more effective than the lipid-soluble antioxidant, vitamin E, on the inhibition of iron-induced morphological transformation. Furthermore, the protection by EGCG on iron-mediated cell transformation occurred at lower concentrations of EGCG compared to vitamin E. This is likely a result of differential bioavailability or potency of these antioxidants to the cells since it has been previously demonstrated that EGCG inhibited oxidative DNA damage (peroxynitrite-mediated 8-oxodeoxyguanosine formation) more efficiently than vitamin C or glutathione, suggesting that EGCG is an effective antioxidant in both hydrophilic and hydrophobic cell compartments (Fiala et al., 1996).

The formation and persistence of DNA strand breaks and DNA damage including sugar and base damage by hydrogen peroxide and iron through the Fenton reaction has been demonstrated in several cell systems (Brawn and Fridovich, 1981; Henle et al., 1996; Imlay and Linn, 1998; Imlay et al., 1988; Lesko et al., 1980). Importantly, several lines of evidence support a role for iron-induced DNA damage in the transformation/carcinogenesis process. Provided that that damage to DNA is severe enough to surpass cellular repair capabilities, DNA damage can accumulate and, if the cell undergoes DNA replication, may result in mutation (Imlay et al., 1988). Additionally, the formation of OH8dG (a major form of oxidative DNA damage) occurs upon the interaction of hydroxyl radicals with cellular DNA. Since OH8dG induces GC $\rightarrow$ TA transversions, OH8dG formation has been correlated with carcinogenicity and mutation (Floyd, 1990; Kasai, 1997). The present results, showing an increase in OH8dG by iron at concentrations that induced morphological transformation, support an association between OH8dG and cell transformation. Furthermore, previous studies have shown that cellular levels of OH8dG produced by photoactivated methylene blue in SHE cells directly correlated with cellular transformation (Zhang et al., 2000).

The induction of DNA strand breaks by iron was examined by single cell gel electrophoresis (comet assay; Rojas et al., 1999). A significant increase in DNA strand breaks was seen following treatment with 2.5 and 5.0 µg/ml of ferrous sulfate treatments at 24 h. Since the induction of cellular transformation by iron required more than 24 h treatment (data not shown), these results indicate that the induction of DNA damage by ferrous sulfate may be related to cellular transformation. Several other in vitro studies have similarly reported the formation of DNA strand breaks by the Fenton reaction (Lloyd and Phillips, 1999; Lloyd et al., 1998; Toyokuni and Sagripanti, 1999).

In addition, the formation and accumulation of OH8dG has been suggested as a mechanism for the liver toxicity associated with iron overload in a rat model for hemachromatosis (Kang et al., 1998). As this human genetic disease is associated with an increase in liver neoplasia, the findings of the present studies, reporting both a significant increase of oxidative DNA damage (OH8dG formation) and the induction of DNA damage by ferrous sulfate treatment, further support the role of iron as a mediator of the carcinogenicity of 2-butoxyethanol. Other studies have reported a positive correlation between accumulation of OH8dG and DNA strand breaks (Lloyd et al., 1998; Toyokuni and Sagripanti, 1999). Since in the present studies iron-induced DNA strand breaks and OH8dG formation were prevented by coexposure to antioxidants, the involvement of oxidative stress and subsequent DNA damage in ferrous sulfate-induced morphological transformation is further substantiated.

Ferrous sulfate-induced hydroxyl radicals, produced from hydrogen peroxide via the Fenton reaction, may not only damage DNA damage but may also produce lipid peroxidation and protein modification. Peroxidation of the cell membrane and/or damage to membrane proteins may result in a loss of membrane integrity, altered membrane fluidity, or modify cellular functions and induce cell death (Richter, 1987). These additional oxidative lesions may result from iron overload and contribute to iron-related carcinogenesis and should not be excluded as being causally related to the transformation/carcinogenesis process.

In summary, the present results showed that ferrous sulfate, but not 2-butoxyethanol or 2-butoxyacetic acid, induced morphological transformation in SHE cells. Iron treatment also resulted in an increase in oxidized DNA damage and single strand breaks in DNA. Furthermore, coincubation with antioxidants was associated with a reduction in the observed iron-induced DNA damage and cellular transformation. Considering that hemolysis caused by 2-butoxyacetic acid results in iron overload in the liver of treated rodents, these in vitro data support that the hepatocarcinogenicity of 2-butoxyethanol involves iron accumulation and resulting oxidative stress and damage in the liver.

Footnotes

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